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Association of Arthritis With a Gene Complex Encoding C-Type Lectin–like Receptors

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Objective. **To identify susceptibility genes in a rat model of rheumatoid arthritis (RA) and to determine whether the corresponding human genes are associated with RA.**

Methods. **Genes influencing oil-induced arthritis (OIA) were position mapped by comparing the susceptibility of inbred DA rats with that of DA rats carrying alleles derived from the arthritis-resistant PVG strain in chromosomal fragments overlapping the quantitative trait locus *Oia2*. Sequencing of gene complementary DNA (cDNA) and analysis of gene messenger RNA (mRNA) expression were performed to attempt to clone a causal gene. Associations with human RA were evaluated by genotyping single-nucleotide polymorphisms (SNPs) in the corresponding human genes and by analyzing frequencies of alleles and haplotypes in RA patients and age-, sex-, and area-matched healthy control subjects.**

Results. **Congenic DA rats were resistant to OIA when they carried PVG alleles for the antigen-presenting lectin-like receptor gene complex (*APLEC*), which encodes immunoregulatory C-type lectin–like receptors. Multiple differences in cDNA sequence and mRNA expression precluded cloning of a single causal gene. Five corresponding human *APLEC* genes were identified and targeted. The SNP rs1133104 in the dendritic cell immunoreceptor gene (*DCIR*), and a haplotype including that marker and 4 other SNPs in *DCIR* and its vicinity showed an indication of allelic association with susceptibility to RA in patients who were negative for antibodies to cyclic citrullinated peptide (anti-CCP), with respective odds ratios of 1.27 (95% confidence interval [95% CI] 1.06–1.52; uncorrected $P = 0.0073$) and 1.37 (95% CI 1.12–1.67; uncorrected $P = 0.0019$). Results of permutation testing supported this association of the haplotype with RA.**

Conclusion. **Rat *APLEC* is associated with susceptibility to polyarthritis, and human *APLEC* and *DCIR* may be associated with susceptibility to anti-CCP–negative RA.**

Rheumatoid arthritis (RA) is a chronic, systemic, and clinically heterogeneous disease characterized by symmetric inflammation in the peripheral joints, leading to cartilage destruction and bone erosion. It affects several ethnic groups, with ~1% prevalence in Western countries, and occurs predominantly in women. Epidemiologic data indicate that the disease evolves from complex interactions of multiple environmental and genetic factors, most of which remain unidentified. The genetic contribution is supported by familial clustering of the disease and by higher disease concordance rates in monozygotic twins than in heterozygotic twins (1). The

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major histocompatibility complex (MHC) is estimated to account for ~30% of the genetic influence (2), with a major role for highly polymorphic class II genes (3). This association with class II MHC is believed to reflect the contribution of autoimmune processes to RA, in that antigen-presenting cells (APCs) use class II MHC proteins to display autoantigens to CD4+ T cells and thereby provide a basis for both cellular and humoral autoimmunity.

To facilitate the identification of genetic risk factors other than the MHC, several research groups have pursued large-scale genetic dissections of induced polygenic RA in rodent models. In the present study, we aimed to determine whether this comparative genetic approach can identify genes that are associated with human RA. To accomplish this, we carried out positional identification of risk genes in rodents, followed by association testing of the corresponding human genes.

In previous studies, we initiated genetic dissection of oil-induced arthritis (OIA) in inbred DA rats. This experimental polyarthritis is mediated by CD4+ T cells, and genetic predisposition is only partly explained by the MHC, which was the quantitative trait locus (QTL) first identified and therefore designated *Oia1*. Linkage analysis of F_2 intercrosses between the DA rat strain and MHC-identical, but arthritis-resistant, rat strains subsequently led to the identification of a novel QTL for susceptibility to OIA (4,5), designated *Oia2*. Reciprocal transfer between strains of chromosome 4 intervals containing the QTL could confer both arthritis susceptibility and arthritis resistance (6), and a panel of recombinant strains allowed further mapping of the QTL to a 1.2-Mb interval on cytogenetic band 4q42. Several different types of genes were predicted in the mapped *Oia2* chromosomal interval (6), including genes encoding members of the C-type lectin superfamily (CLECSF, or CLSF). These candidate disease genes were subsequently cloned, together with novel similar genes, and were shown to be arranged in tandem within an evolutionarily conserved gene complex, designated the antigen-presenting lectin-like receptor complex (*APLEC*), since the encoded receptors are preferentially expressed on APCs and neutrophils (7).

In the present study, we first positioned mapped *Oia2* to rat *APLEC* and determined that no *APLEC* gene could be singled out as being causal in this arthritis model. We then targeted the human *APLEC* genes in a case–control association study of RA. Associations were determined both before and after stratification for the presence or absence of antibodies to cyclic citrullinated peptide (anti-CCP), which defines clinically and genetically distinct subsets of patients with anti-CCP–positive RA and anti-CCP–negative RA (8,9). Our results indicate an association between anti-CCP–negative RA and the human *APLEC* dendritic cell (DC) immunoreceptor gene, *DCIR*, on human chromosome 12p13.31.

MATERIALS AND METHODS

Animal rearing, arthritis induction, and disease evaluation. Inbred PVG.1AV1 rats originating from Harlan UK (Oxon, UK) and inbred DA and LEW.1AV1 rats originating from Zentralinstitut für Versuchstierzucht (Hannover, Germany) were used in the animal experiments. Congenic strains were derived by marker-assisted transfer of selected chromosome 4 intervals from PVG.1AV1 rats onto the DA background through 13–15 backcross generations for the R8, R10, and R11 strains, as previously described (6). Heterozygous congenic R17 rats were derived from an intercross between the DA and R11 strains (15 backcross generations), followed by a backcross of 1 selected recombinant rat onto the DA background, resulting in 17 backcross generations. The strains contained PVG alleles on the DA background, as indicated in Figure 1.

Animals were bred, kept, and used at the Center for Molecular Medicine, Karolinska Institutet, Stockholm, under specific pathogen–free conditions, as determined by a health monitoring program at the National Veterinary Institute in Uppsala. They were reared in a climate-controlled environment with 12-hour light/dark cycles, were housed in polystyrene cages containing wood shavings, and had free access to standard rodent nourishment.

Arthritis was induced by intradermal injection of 200 μl of Freund's incomplete adjuvant (Difco, Detroit, MI) at the base of the tail of anesthetized animals. All animals were matched for age and sex. The development of arthritis was monitored using a macroscopic scoring system, with scores ranging from 0 to 4 per limb, yielding a total macroscopic score of arthritis of 0–16 per animal, as detailed previously (6). All procedures involving animals were performed according to the guidelines provided by the central Board for Animal Experiments at the Swedish Department of Agriculture, and were approved by the ethics committees of Stockholm North.

Genotyping with microsatellite markers. Rat genomic DNA was used for polymerase chain reaction (PCR) amplification of polymorphic tandemly repeated sequences using ^{33}P-γ-ATP to label a single primer in each pair, which allowed determination of the genotypes by autoradiography following size separation of the PCR product by gel electrophoresis (6). PCR primer pairs were purchased from Proligo (Paris, France) based on public sequence information from the Rat Genome Database (www.rgd.mcw.edu) for the *D4Rat* and *D4Got* markers, or based on our own identification of sequences bordering tandem repeats for the *D4Kiru* markers. The latter primer pairs were as follows: for *D4Kiru90*, forward CTCCAGAAAGACGTGAAGATCA, reverse AGAAGCAGGCTAATCAGTGTGA; for *D4Kiru151*, forward AGCTTTTGCAGAGATGGTTTTC, reverse AAAGCCCTTATTACCCCAGAGT; for *D4Kiru12*, forward AGGAGGGTAGGGTAAATGAAGTAAT, reverse GTCTAGCTGGGACGTT-

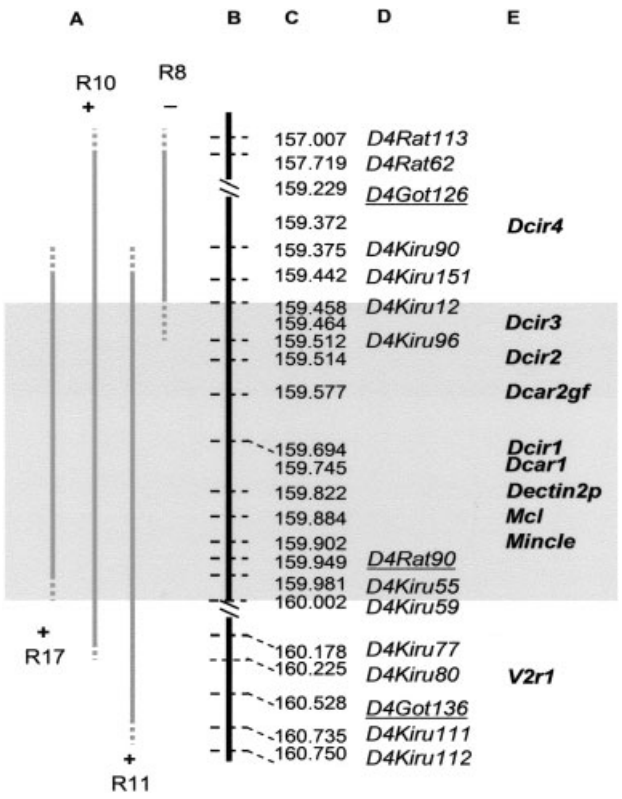

Figure 1. Mapping of the *Oia2* region (indicated by shaded area) in recombinant rat strains. **A,** Outer borders of the region are defined by the influence (+) or noninfluence (−) on arthritis susceptibility (6) of intervals transmitted from the PVG to DA background in the recombinant strains R8, R10, R11, and R17. Intervals are depicted as shaded vertical lines, with extending broken lines indicating nondetermined genotypes at crossover points. **B,** Rat chromosome 4 is depicted as a solid vertical line, with **C,** chromosomal positions (in kb) for **D,** microsatellite markers and **E,** genes. The *Oia2* genes listed in **E** are indicated from the microsatellite markers *D4Kiru90* to *D4Kiru59*. Underlined markers indicate reference markers previously used to define the *Oia2* interval. Genes listed in **E** are members of the C-type lectin superfamily. Positions are based on Rat Genome Sequencing Consortium assembly 3.4. These genes are *Dcir1–4* (dendritic cell [DC] immunoreceptor genes), *Dcar1* and *Dcar2gf* (DC-activating receptor 1 gene and DC-activating receptor 2 gene fragment), *Dectin2p* (DC-associated C-type lectin 2 pseudogene), *Mcl* (macrophage C-type lectin gene), *Mincle* (macrophage-inducible C-type lectin gene), and *V2r1* (vomeronasal type 2 pheromone receptor gene).

GCTT; for *D4Kiru96*, forward TCTCACTCCTTATCATTTCATCCA, reverse CCCAATAGACAGGGTTAATGATTC; for *D4Kiru55*, forward TGCACCTGAGCATGTAAGTGTAT, reverse CCTGAACAGTCCTGACATAAACC; for *D4Kiru59*, forward GCCTCATTGTTAGCCTTGTGTTA, reverse TGAAGTTAGAAACTGGCCTTCTG; for *D4Kiru77*, forward TATTTTGGGGAAGATGAAGAAACT, reverse TTCAGGAACATCTCTAACTTCAGC; for *D4Kiru80*, forward ACTGTGGAAGAGATTCCAGATGA, reverse GCAATGTCTCTGTCTTCAACTCC; for *D4Kiru111*, forward CAGACTTATGGTAGAGATTGGCTATG, reverse TACCATTATTGTGAGGTGAAGAGTTT; and for *D4Kiru112*, forward TCCTTCATACACATGGTACACATAAA, reverse CATTCCCTGCTTAAACTATTCTTTTT.

Exon trapping. P1-derived artificial chromosome (PAC) clone 371 A15, from a female BN rat library (10), was identified by PCR on superpooled clones, using primers specific for the marker *D4Rat90*, followed by hybridization to array filters using ^{32}P-labeled DNA probe (11). PAC DNA was isolated using a modification of a standard protocol. Briefly, 200 ml of Luria-Bertani (LB) culture medium was used overnight with 15 ml each of buffers P1, P2, and P3; elution from the column was carried out twice, each time with 7.5 ml elution buffer heated to 65°C (Plasmid Maxi kit; Qiagen, Hilden, Germany). Individual PAC clones were analyzed by pulsed-field gel electrophoresis and fingerprinting (11). PACs were digested with *Bam* HI and *Bgl* II (New England Biolabs, Beverly, MA), and the resulting fragments were ligated into pSPL3b (PubMed identification no. 7665076), electroporated into *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA), and cultured on LB agar plates to ensure equal expansion of individual clones. Bacteria were harvested from the plates and pooled, and plasmid DNA was isolated (Qiagen) and used to transfect 293T cells using Lipofectamine and Opti-MEM (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

Cells were harvested 30 hours after the start of transfection. Total RNA was isolated by a guanidinium isothiocyanate/CsCl centrifugation protocol (11), and first-strand complementary DNA (cDNA) was generated using SuperScript II reverse transcriptase and RNAse H (standard protocol; Invitrogen) and a primer specific for the 3′ vector–derived exon (5′-ATCTCAGTGGTATTTGTGAGC-3′). Reverse transcription–PCR (RT-PCR) was then performed using this primer together with a primer specific for the 5′ vector–derived exon (5′-TCTGAGTCACCTGGACAACC-3′) and using DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland). The PCR product was purified (Wizard PCR Preps; Promega, Madison, WI), digested with *Bst* XI to degrade products lacking trapped exons or resulting from cryptic splicing, subjected to a second PCR step with a pair of nested primers (5′-CUACUACUACUAGTGAACTGCACTGTGACAAGCTGC-3′/5′-CUACUACUACUACACCTGAGGAGTGAATTGGTCG-3′), and cloned into pAMP10 using uracil *N*-deglycosylase (standard protocol; Invitrogen). The ligated plasmids were electroporated into XL-1 Blue (Stratagene) and plated on LB agar plates. Clones were selected by blue–white screening and sorted according to exon size by PCR (primers 5′-GTGAACTGCACTGTGACAAGCTGC-3′/5′-CACCTGAGGAGTGAATTGGTCG-3′) followed by electrophoresis. DNA (Qiagen) was purified and the inserts of 24 individual clones (GATC Biotech, Constance, Germany) from each PAC clone were sequenced.

Gene cloning and sequence analyses. Cervical lymph nodes from PVG, DA, and LEW.1AV1 rats were collected. Total cellular RNA was isolated from the lymph nodes as previously described (12). First-strand cDNA synthesis was carried out with Moloney murine leukemia virus reverse transcriptase RNase H^- and oligo-d$(T)_{15}$ (Promega), as previously described (12). Gene products were amplified by PCR

using a high-fidelity DNA polymerase, *Pfu Turbo* (Stratagene), and gene-specific primers. The gene products were cloned into pCR-2.1 TOPO (Invitrogen). For each cDNA, 3 clones were selected for sequencing (MediGenomix, Martinsried, Germany). The sequences were analyzed using software supplied by the Norwegian EMBNet node at the Biotechnology Centre in Oslo, Norway. In humans, DNA from 20 individuals was resequenced for exons of the DC-associated C-type lectin 2 (*DECTIN2*) with flanking regions in both directions (Cybergene, Huddinge, Sweden).

Gene expression analyses. Tissue samples were obtained from the cervical lymph nodes of naive DA rats and R17 rats. Cells from the lymph nodes were harvested and washed, and total RNA was extracted according to the instructions in the RNeasy Mini Kit (Qiagen). Samples were incubated with DNase for 30 minutes at room temperature, to avoid genomic DNA contamination (Qiagen RNase-free DNase set). RT was performed with 10 μl of total RNA, a random hexamer primer (0.1 μg/ml; Life Technologies, Gaithersburg, MD), and SuperScript reverse transcriptase (200 units/μl; Life Technologies). All primers were designed using Primer Express software (PerkinElmer, Norwalk, CT) and were constructed over the exon–exon boundaries of the cDNA sequence (Entrez Nucleotide, accessed at the National Center for Biotechnology Information Web site http://www.ncbi.nlm.nih.gov/geo/), to avoid amplification of contaminating genomic DNA.

Primer pairs were purchased from Cybergene. The housekeeping gene *GAPDH* was used as an endogenous control. The primers used for this experiment were as follows: for *Dcir1*, 5′-CCATAGCAAGGAAGAACAGGACTT and 3′-TGAATCCCAGAGCCCTATAAAATAA; for *Dcir2*, 5′-CACAAAAAATGTTTCACTCACAGAAGATA and 3′-AGTAAAGTAGCAGTAGGAGCCAAATGA; for *Dcir3*, 5′-AGCCAGGAAGAGCAGGATTTC and 3′-TGATCGACCCATTGCCATT; for *Dcir4*, 5′-CATTCGTCCGTGGAAGACAAA and 3′-TGCAGAGTCCCTGGAAGTGAA; for the DC-activating receptor 1 gene (*Dcar1*), 5′-TGCTCATCTGTTGGTGATCCA and 3′-TGTAAAATAACCCCAACGAGTGTCTA; for the macrophage-inducible C-type lectin gene (*Mincle*), 5′-TTTCACAGAGTCCCTGAGCTTCT and 3′-TCCCTCATGGTGGCACAGT; for the macrophage C-type lectin gene (*Mcl*), 5′-CACAAGGCTAACATGCATCCTAGA and 3′-GCAAAGTAACAGTTAGACTGGAATGCT; and for *GAPDH*, 5′-TCAACTACATGGTCTACATGTTCCAG and 3′-TCCCATTCTCAGCCTTGACTG. All samples were analyzed individually. Amplification and quantitative analyses were performed using an ABI PRISM 7700 Sequence System (PerkinElmer) and the SYBR Green method (QuantiTect SYBR Green; Qiagen). Relative quantification of messenger RNA (mRNA) levels was carried out using a standard curve method (2001 User Bulletin 2; PerkinElmer, and Applied Biosystems, Branchburg, NJ). Standard curves were generated using 5 serial dilutions (1:1, 1:5, 1:25, 1:125, and 1:625) of mixed cDNA samples from 4 DA rats and 5 R17 rats. Individual samples were run in duplicate, and the relative quantity of mRNA for each target was calculated as the quotient between target and GAPDH mRNA.

Human subjects. The human study population consisted of 1,889 patients with RA and 1,069 healthy control subjects ages 16–82 years, of whom 2,148 were female and 810 were male. Among all subjects, 630 were anti-CCP negative and 991 were anti-CCP positive, while 518 were rheumatoid factor (RF) negative and 992 were RF positive. The anti-CCP status was determined as previously described (9).

All patients with RA were identified by rheumatologists as a part of a surveillance program for early RA, i.e., the diagnosis was made between 2 months and 12 months after the first appearance of symptoms of the disease. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA (13). The control subjects were randomly selected from the same study base as the patients, and were matched for age, sex, and residential area. All subjects gave their informed consent to participate in the study, and the regional ethics committee of Karolinska Institutet approved the study protocol. Additional characteristics of these subjects as well as the procedures and rheumatology departments involved in the collection of data have been described previously (14).

Single-nucleotide polymorphism (SNP) genotyping. Dynamic allele-specific hybridization (DASH) of SNPs was performed as previously described (15–17). Two PCR primers and 1 DASH probe per target SNP were designed using custom DFold software (18) provided by DynaMetrix (Hertfordshire, UK). These oligonucleotides were purified by high-performance liquid chromatography (Biomers, Ulm, Germany). The DASH PCRs entailed amplification of short genomic fragments spanning the variant of interest, with 1 of the primers carrying a 5′-biotin label. Amplifications were performed in 5-μl volumes, containing 1–2 ng genomic DNA, 0.38 μM biotinylated primer, 0.75 μM nonbiotinylated primer, 0.03 units AmpliTaq Gold (PerkinElmer Applied Biosystems, Foster City, CA), 10% dimethylsulfoxide, 1× AmpliTaq Gold Buffer including 1.5 mM of $MgCl_2$ (PerkinElmer Applied Biosystems), and 0.2 mM each dNTP. Thermal cycling was conducted on a Multi-Block System 384-well thermal cycler (Thermofisher Scientific, Basingstoke, UK) as follows: 1 cycle for 10 minutes at 94°C, 35 cycles for 15 seconds at 94°C, and 30 seconds at annealing temperature. To verify successful amplification, 0.5 μl of several randomly chosen samples was examined on a 3.0% low-melt agarose gel.

DASH analysis of the PCR product was conducted on membrane macroarrays, using the DASH-2 protocol (15). Briefly, this involved transferring samples to the membrane array by centrifugation (19) or robotic gridding. The resulting individual arrays, with up to 9,600 distinct features, were rinsed in 0.1M NaOH to denature the PCR products, and then exposed to 2 ml HE buffer (0.1M HEPES, 10 mM EDTA, pH 7.9) containing 4 nmoles of suitable probe that was end-labeled with ROX. After heating to 85°C and cooling to room temperature, the membrane was briefly rinsed in HE buffer. The array was then soaked for up to 3 hours in 40 ml HE buffer containing SYBR Green I dye at 1:20,000 dilution. Using a DASH-2 device (DynaMetrix), the membrane was taken through a DASH heating ramp (heating at 3°C/minute from room temperature to 85°C), while fluorescence from the ROX acceptor dye on the probe was monitored. Data were collected at intervals of 0.5°C.

Fluorescence changes occurring in parallel with changing temperature (DNA melting profiles) were used to distinguish different alleles, and this was done using DASH-2 device software, which uses negative derivatives of fluorescence

against temperature to reveal peaks of the denaturation rate (target–probe melting temperatures), and thereby DNA samples are automatically assigned into genotype groups. All samples positive for the minor allele of rs1133104 and samples that were not successfully genotyped for this marker by DASH (in total, 6.7%) were subjected to further genotype analyses using PCR-based restriction endonuclease mapping, which involved use of the primers 5′-GCCGGAAATGCAGATGGAGGAGAAGCTACATGCTGA-3′ and 5′-AACCAGATAAAAATTAAGGGAGGCTCATGTTCTCTAC-3′, and *Nsp* I for cleavage.

A TaqMan allelic discrimination analysis (5′ nuclease assay) was performed according to ABI standard protocols (PerkinElmer). The assay design and quality control were provided by Applied Biosystems. Genotyping was performed in a 384 format using an ABI 7700 GeneAnalyser, with end-point measurements after 35 cycles. An experienced operator checked automatic genotype assignments, and data considered to be major outliers were removed from the analysis. Due to the logistics of the study, the first 24 markers were genotyped by DASH in samples from 1,285 patients with RA and 709 healthy controls (between 1,700 and 1,983 successfully identified genotypes per SNP), while the next 11 markers were genotyped by TaqMan in samples from 1,889 patients with RA and 1,069 healthy controls (between 2,257 and 2,766 successfully identified genotypes per SNP; SNPs 8–12, 16, 17, 20, 22, 26, and 27 [see Figure 5]). The SNPs rs4573763, rs7302963, rs4604965, and rs7307734 were genotyped both by TaqMan and by DASH. The marker rs1133104 was genotyped by TaqMan as well as by DASH and restriction endonuclease mapping. Discrepant genotype rates ranged from 0.1 to 0.4, and these discrepant genotypes were excluded from further analysis.

Gene designations and accession numbers. Genes that were identified in the human subjects had the following common current designations in gene databases: for *DLEC*, BDCA2, CLECSF7, CLECSF11, and CLEC4C (in HUGO), and CLEC7 (in Ensembl); for *DCIR*, CLECSF6, and CLEC4A (in HUGO), and CLEC6 (in Ensembl); for *DECTIN2*, CLECSF10 and CLEC6A (in HUGO); for *MCL*, CLECSF8, and CLEC4D (in HUGO), and CLEC8 (in Ensembl); and for *MINCLE*, CLECSF9, and CLEC4E (in HUGO), and CLEC9 (in Ensembl). In the rat model, the identified genes had the following common current designations: for *Dcir1*, Clecsf6; for *Dectin2*, Clecsf10; for *Mcl*, Clecsf8 and Clec4d; and for *Mincle*, Clecsf9. There are no current Clecsf designations for *Dcir2, Dcir3*, *Dcir4*/*Clec4a1*, *Dcar2gf*, and *Dcar1*/*AplrA1*. For the DA rat cDNA, the associated accession numbers were as follows: for *Mincle*, AY494064; for *Mcl*, AY494065; for *Dcar1*, AY494066; for *Dcir1*, AY494060; for *Dcir2*, AY494061; for *Dcir3*, AY494062; and for *Dcir4*, AY494063. For the LEW.1AV1 rat cDNA, the associated accession numbers were as follows: for *Mincle*, AY581049; for *Dcar1*, AY581050; for *Dcir1*, AY581051; and for *Dcir2*, AY581052. The sequences of PVG rat cDNA have previously been described (7).

Statistical analysis. Evaluation of allele frequencies, linkage disequilibrium (LD), estimation of haplotype frequencies, haplotype associations, and permutation tests (of 10,000 permutations) were performed using Haploview software, version 3.2 (http://www.broad.mit.edu/mpg/haploview/index.php) (20). Genotype frequencies were compared using chi-square test, and odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated using StatCalc (EpiInfo, version 6; Centers for Disease Control and Prevention, Atlanta, GA). Rat arthritis scores and gene expression data were evaluated by Mann-Whitney U test using Statistica (version 6; StatSoft, Tulsa, OK).

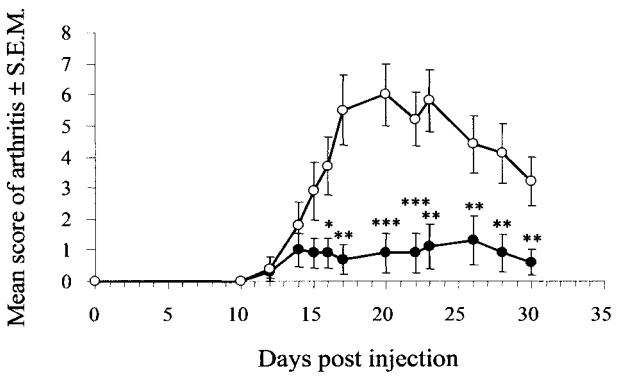

Figure 2. Influence of the *Oia2* region on the development of oil-induced arthritis in rats. Results are the mean ± SEM macroscopic arthritis scores following injection of arthritogenic oil in DA rats (open circles) and in DA rats heterozygous for PVG alleles within *Oia2*, i.e., R17 strain (solid circles). Each group consisted of 5 males and 5 females (n = 10). Differences in arthritis scores between the 2 strains were significant from day 16 postinjection until the end of the experiment, as calculated by Mann-Whitney U test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

RESULTS

High-resolution position mapping of a rat chromosomal interval that regulates arthritis. A set of 16 congenic DA strains that are heterozygous for recombinant rat chromosome 4 intervals (derived from the arthritis-resistant PVG strain) has been used previously to map *Oia2* to a 1.2-Mb interval (6) between the microsatellite markers *D4Got126* and *D4Got136.* In the present study, we produced a new recombinant strain, designated R17, which contains a fraction of the 1.2-Mb interval from the PVG rats. The transferred PVG alleles confer markedly reduced susceptibility to arthritis, as was observed in heterozygous R17 rats compared with DA rats (Figure 2); this could be attributed mostly to a decrease in arthritis incidence in the R17 strain (arthritis in 3 of 10 R17 rats versus 10 of 10 DA rats; $P = 0.003$ by Fisher's exact test).

To further narrow our investigation of *Oia2* through definition of the exact recombination points in the recombinant strains used, we developed novel markers by identifying tandem repeats in the interval genomic sequence, designing the corresponding PCR primer

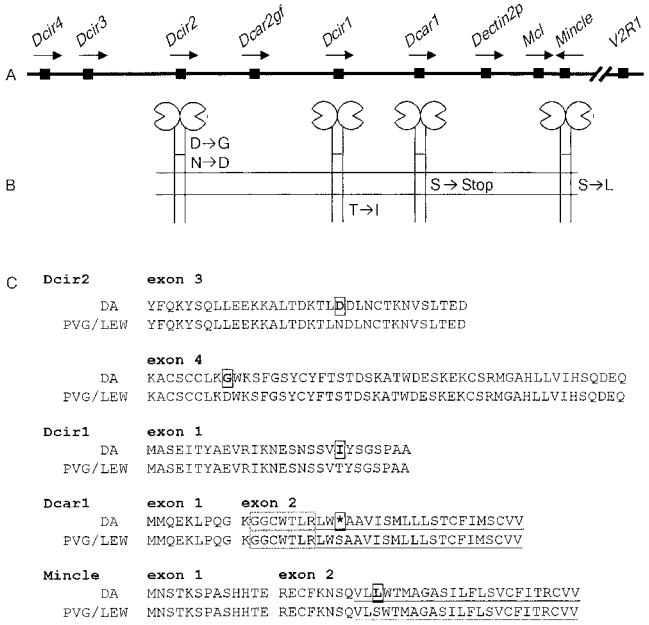

Figure 3. The rat C-type lectin–like receptor gene complex (*APLEC*). **A,** Genes identified in the *APLEC* are defined by their orientations and relative positions. **B,** Proteins are depicted iconically, with N-terminal starts at the bottom (cytoplasmic domain) and the C-type lectin fold on top. The double line indicates the cell membrane. The receptors are presumed to form disulfide-linked homodimers or heterodimers. Letters indicate nonsense and substitution (PVG→DA) mutations. **C,** Amino acid sequence alignments are shown for the exons with missense (*Dcir2*, *Dcir1*, and *Mincle*) or nonsense (*Dcar1*) mutations. Boxes indicate the sites in which the DA sequences deviate from the PVG and LEW sequences. Transmembrane regions are underlined. The asterisk indicates a nonsense mutation. The dotted boxes indicate possible extension of the transmembrane domain in *Dcar1* (includes an arginine [R], with the possibility of activating function). Positions are based on the Rat Genome Sequencing Consortium assembly 3.4.

pairs, and testing the PCR products for polymorphisms between DA and PVG. Altogether, 19 informative *Kiru* microsatellite markers were used to genotype the recombinant strains that delimit *Oia2*, thereby defining the DNA regions within chromosome 4q42 that appear to either have (strains R10, R11, and R17) or not have (strain R8) influence on arthritis susceptibility. We thus reduced *Oia2* to a 544-kb interval with outer boundaries between *D4Kiru12* and *D4Kiru59*, and also established that the R17 strain contains PVG alleles within a 627-kb interval that harbors the mapped *Oia2* interval (Figure 1).

Exclusive presence of *APLEC* genes in the position-mapped rat chromosomal interval. The mapped arthritis-regulating *Oia2* interval corresponds almost completely to the 535-kb gene complex designated *APLEC* (7), which contains 7 lectin-like receptor genes, i.e., *Mincle* and *Mcl*, as well as *Dcar1*, *Dcir1–Dcir4*, the *Dectin* pseudogene *Dectin2p*, and the gene fragment named *Dcar2gf* (related to *Dcar1*). All of these *APLEC* genes are located within *Oia2*, except that the centromeric end lacks *Dcir4* and its promoter region and lacks the 3 first exons of *Dcir3*; therefore, these are not dominant genetic factors in our experimental system (Figure 1). The telomeric end of *Oia2* has an extraordinarily high density of long and short interspersed nuclear repeat elements (LINEs and SINEs, respectively) but contains no predicted genes other than the lectin-like receptors.

In addition to relying on gene prediction analyses, we searched for evidence of other genes by performing exon trapping on a PAC clone that was identified by the presence of the *D4Rat90* marker mapped inside *Oia2* (Figure 1). Exons were trapped between chromosomal positions 159,880–160,010 kb, thus overlapping the telomeric end of *Oia2* (Figure 1). Only genes that have already been predicted in the rat genome (see the Ensembl database at http://www.ensembl.org) were identified, e.g., 4 of the 6 *Mcl* exons were trapped, as well as exons from the vomeronasal type 2 pheromone receptor gene (*V2r1*) located telomeric of *D4Kiru59*, and outside *Oia2* according to the rat genome sequence. In addition, many noncoding sequences were trapped, all of which were from LINEs and SINEs. This strongly suggests that lectin-like receptor–encoding genes are responsible for regulating arthritis, and that the congenic rat strain R17 is specific for both *APLEC* and *Oia2*.

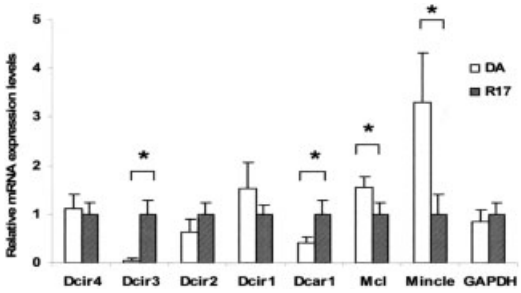

Figure 4. Gene mRNA expression levels in cervical lymph nodes from naive DA rats and R17 rats. Expression of mRNA was determined by quantitative reverse transcription–polymerase chain reaction on individual samples. Differences in mRNA levels were found for *Dcir3*, *Dcar1*, *Mcl*, and *Mincle,* as calculated by Mann-Whitney U test (∗ = $P < 0.05$). Results are expressed as the mean and SD target gene quantity in 5 R17 rats and 4 DA rats, normalized to levels of the *GAPDH* housekeeping gene, and with mRNA expression in R17 rats set to unity (1.0).

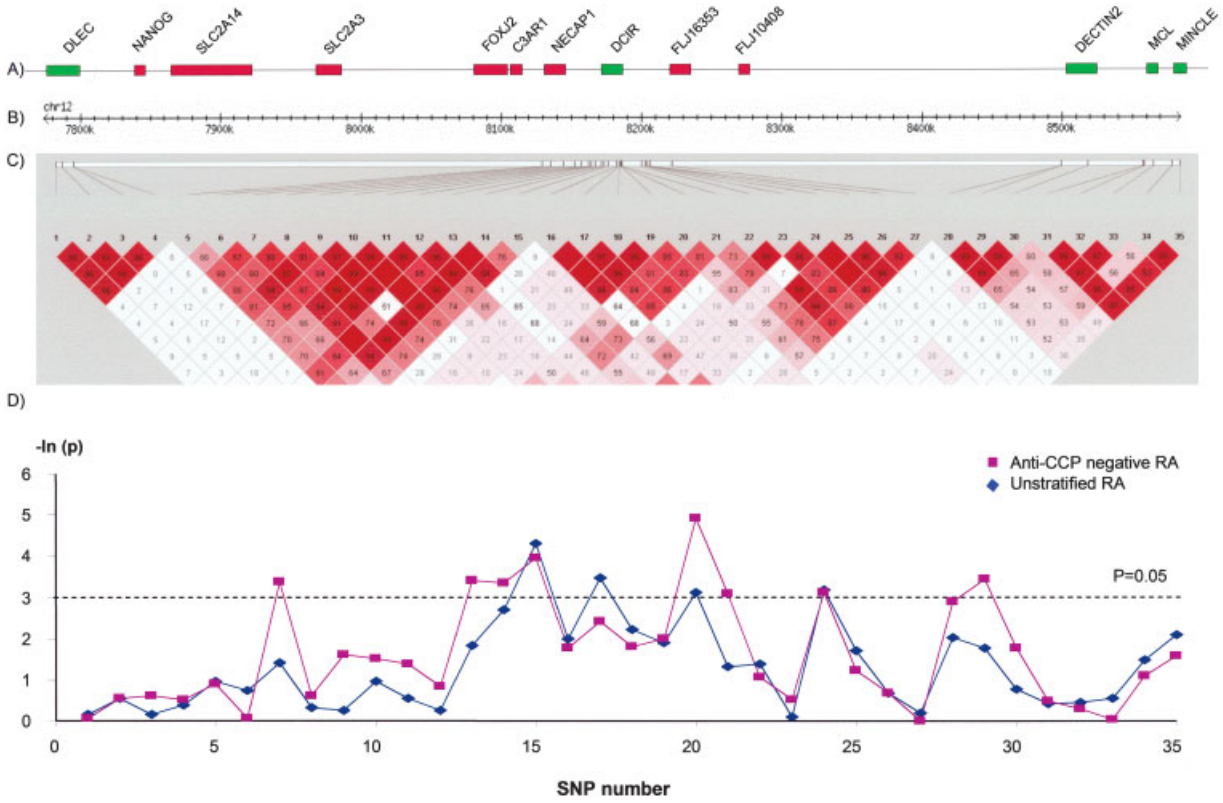

Figure 5. Location and linkage disequilibrium (LD) analyses of single-nucleotide polymorphisms (SNPs). **A,** Genes are identified along with their positions and size in scale; green boxes indicate the C-type lectin–like receptor–encoding genes, and red boxes indicate other genes. **B,** A scale of human chromosome 12q13.31. **C,** Positions of analyzed SNPs and representation of the extent of LD between named markers. **D,** Representation of *P* value (−ln) for allelic associations with rheumatoid arthritis (RA) and anti–cyclic citrullinated peptide (anti-CCP)–negative RA for all analyzed SNPs, listed in the same order as in Table 1.

Comparisons of sequences and mRNA expression levels of the position-mapped rat *APLEC* genes between arthritis-resistant and arthritis-susceptible rat strains. To possibly identify the arthritis-regulating gene, exons of all *APLEC* genes were sequenced as cDNA derived from the differentially susceptible DA and PVG strains (Figure 3), and gene expression levels in DA and R17 rats were determined by quantitative RT-PCR of mRNA from lymph nodes (Figure 4), which is a crucial site for interaction between APCs and T cells (21). As summarized in Figures 3 and 4, missense mutations or differences in mRNA expression levels between the DA and R17 strains were detected for all genes or gene products except Dcir4. With the possible exception of the D110G substitution in the β0 loop of Dcir2, the amino acid substitutions observed in Dcir1, Dcir2, and Mincle were conservative and in presumed innocuous sites (N88D in the stalk of Dcir2; T31I and S24L substitutions in the cytoplasmic domains of Dcir1 and Mincle, respectively). For Dcar1, the DA allele carried a predicted nonsense mutation in the cytoplasmic region at amino acid position 20 (PVG→DA at S20stop), and gene expression was reduced, possibly due to nonsense decay (22). Differences in gene expression levels in lymph nodes were also recorded for Dcir3, Mcl, and Mincle.

Genes exhibiting differences in cDNA sequence between the PVG and DA strains were also sequenced in the LEW.1AV1 strain, from which arthritis resistance can also be transmitted to the DA strain (5,6,23) by transferral of chromosome 4 intervals that harbor *Oia2.* The predicted amino acid sequences for Dcar1, Dcir1, Dcir2, and Mincle were identical between the arthritis-resistant strains LEW.1AV1 and PVG.

Analysis of associations between RA and selected SNPs in human *APLEC* genes. A gene cluster residing on human chromosome 12p13.31 was identified as corresponding to rat *APLEC* (Figure 5). It contained 4

Table 1. Allele frequencies in patients with anti–cyclic citrullinated peptide–negative rheumatoid arthritis (RA) and healthy control subjects, for all single-nucleotide polymorphisms (SNPs) analyzed*

	Allele frequency									
	RA patients				Healthy controls					
Locus, SNP	Allele 1	n	Allele 2	n	Allele 1	n	Allele 2	n	*P*†	OR (95% CI)
DLEC										
(A/C)rs6488608	66	653	34	335	66	898	34	464	0.9353	1.01 (0.84–1.20)
(A/G)rs6488610	66	646	34	338	67	908	33	452	0.5733	0.95 (0.80–1.14)
(A/G)rs7300836	77	764	23	224	76	1,025	24	319	0.5484	1.06 (0.87–1.30)
(A/C)rs7134202	69	677	31	307	68	923	32	439	0.5961	1.05 (0.88–1.26)
NECAP1										
(C/T)rs2192136	70	678	30	292	71	945	29	377	0.4094	0.93 (0.77–1.12)
(C/T)rs2889626	53	518	47	454	53	699	47	617	0.9333	1.01 (0.85–1.19)
(C/G)rs1062836	74	731	26	249	71	922	29	384	0.0347	1.22 (1.01–1.48)
Intergenic										
(A/T)rs7305412	76	909	24	291	75	1,479	25	499	0.5365	1.05 (0.89–1.25)
(A/G)rs2889629	75	824	25	268	73	1,171	27	427	0.2049	1.12 (0.94–1.34)
(A/G)rs2024304	73	880	27	332	71	1,412	29	588	0.2226	1.10 (0.94–1.30)
(C/T)rs2110067	70	837	30	359	68	1,317	32	619	0.2510	1.10 (0.93–1.29)
(A/G)rs1035060	69	829	31	365	68	1,376	32	644	0.4388	1.06 (0.91–1.24)
DCIR										
(A/T)rs4882913	97	964	3	28	98	1,331	2	21	0.0338	0.54 (0.30–1.00)
(A/T)rs2024301	69	683	31	299	65	887	35	469	0.0355	1.21 (1.01–1.45)
(C/T)rs2377422	70	693	30	301	65	899	35	481	0.0193	1.23 (1.03–1.47)
(A/G)rs11043498	90	1,090	10	118	92	1,822	8	166	0.1719	0.84 (0.64–1.09)
(A/G)rs4322490	91	1,074	9	112	92	1,851	8	155	0.0905	0.80 (0.62–1.04)
(C/T)rs4573763	91	1,120	9	116	92	1,899	8	165	0.1659	0.84 (0.65–1.08)
(C/T)rs7302963	89	1,088	11	136	90	1,810	10	190	0.1409	0.84 (0.66–1.07)
(G/T)rs1133104	80	978	20	250	75	1,389	25	451	0.0073	1.27 (1.06–1.52)
(A/G)rs10840759	80	925	20	227	77	1,307	23	387	0.0456	1.21 (1.00–1.46)
Intergenic										
(A/T)rs4589367	65	768	35	408	64	1,224	36	700	0.3411	1.08 (0.92–1.26)
(C/T)rs4604965	64	797	36	443	63	1,306	37	754	0.6112	1.04 (0.89–1.21)
(A/G)rs7307734	97	1,198	3	36	98	2,022	2	38	0.0443	0.63 (0.38–1.02)
(A/G)rs6486850	60	583	40	389	58	762	42	556	0.2984	1.09 (0.92–1.30)
(A/G)rs6486851	63	722	37	426	62	1,196	38	742	0.5140	1.05 (0.90–1.23)
(C/T)rs10840926	56	629	44	485	56	897	44	691	0.9905	1.00 (0.85–1.17)
DECTIN2										
(A/T) SNP 1	81	950	19	220	78	1,332	22	370	0.0557	1.20 (0.99–1.45)
(C/T) SNP 2	82	949	18	215	78	1,335	22	371	0.0325	1.23 (1.01–1.49)
(A/C) SNP 3	83	978	17	194	81	1,383	19	315	0.1683	1.15 (0.94–1.40)
MCL										
(A/T)rs4534636	83	814	17	170	82	1,101	18	243	0.6160	1.06 (0.85–1.32)
(C/T)rs4304840	78	766	22	214	79	1,053	21	285	0.7563	0.97 (0.79–1.19)
(C/T)rs7976134	79	761	21	199	79	1,072	21	282	0.9543	1.01 (0.82–1.24)
MINCLE										
(A/C)rs7307228	58	557	42	409	56	744	44	592	0.3463	1.08 (0.91–1.29)
(A/G)rs4620776	75	742	25	250	72	999	28	379	0.2105	1.13 (0.93–1.36)

* Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated only for the number of chromosomes positive/number of chromosomes negative for a specific allele in the RA patients/healthy control group, using StatCalc. All markers were in Hardy-Weinberg equilibrium in these samples, except for rs7300836 ($P = 0.015$). Values for allele frequency are the percentage of each allele (allele 1 or allele 2) of the total number (n) of alleles. The parentheses in each SNP designation indicate allele 1/allele 2, defined as follows: Allele 1 means A for rs7134202, rs7300836, rs6488610, rs6488608, rs2024304, rs4882913, rs2024301, rs10840759, rs7307734, rs6486850, rs6486851, SNP 1, SNP 3, rs4534636, rs7307228, and rs4620776; C for rs2192136, rs2889626, rs1062836, rs2110067, rs2377422, rs4573763 rs7302963, rs4604965, SNP 2, rs4304840, and rs7976134; G for rs2889629, rs1035060, rs11043498, rs4322490, and rs1133104; T for rs7305412, rs7488293, and rs10840926. Allele 2 means A for rs7305412, rs2889629, rs1035060, rs11043498, rs4322490, and rs7488293; C for rs7307228, rs6488608, rs10840926, SNP 3, and rs7307228; G for rs7300836, rs2024304, rs10840759, rs6488610, rs1062836, rs7307734, rs6486850, and rs4620776; T for rs2192136, rs2889626, rs2110067, rs4882913, rs2024301, rs2377422, rs4573763, rs7302963, rs1133104, rs4604965, rs6486851, SNP 1, SNP 2, rs4534636, rs4304840, and rs7976134.

† *P* values were calculated in Haploview and were not corrected for multiple testing.

homologous genes with conserved relative order and orientation compared with rodents (7). These genes were *DCIR* (24), *DECTIN2* (7), *MCL* (25), and *MINCLE* (26), which are orthologous to rat *Dcir1* and

Dcir2, *Dectin2p*, *Mcl*, and *Mincle*, respectively. A fifth human gene, DC lectin (*DLEC*) (27), was located telomeric to *DCIR* in an inverted orientation. Human *DLEC* and rat *Dcar1* do not appear to have direct orthologs, but they are highly related to each other and also to *DCIR*/*Dcir1–4* and *DECTIN2*/*Dectin2p* (7,28).

In total, 35 SNPs were used to target the human genes and *DCIR* flanking regions. The procedure for marker selection was as follows. First, SNPs in the vicinity of *DLEC*, *DCIR*, *MCL*, and *MINCLE* were identified in SNP public databases, limiting the database search to markers that have been indicated to be valid and located in nonrepetitive DNA. Second, novel SNPs in *DECTIN2* were identified by resequencing the gene (promoter, exons, and flanking intronic regions) in 20 human DNA, yielding 3 SNPs with chromosome positions 8,499,494, 8,499,548, and 8,518,497 bp. Third, all markers were evaluated in a population of 16–96 healthy Swedish individuals, representing 32–192 chromosomes. Markers were positively selected for heterozygosity, and SNPs that demonstrated evidence of copy number variability were excluded from the analysis.

The SNPs were tested for disease association by successfully genotyping them in a case–control study cohort of between 1,700 and 2,766 individuals, comprising patients with well-defined RA along with sex- and age-matched controls recruited from the same population and same geographic area (14). All analyzed SNPs in our study were in Hardy-Weinberg equilibrium. Single-marker tests for allele and genotype associations with RA were first performed before stratification for anti-CCP status (Figure 5). Four markers showed a nominal allelic association, of which 3 were located in *DCIR* (rs2377422 [$P = 0.014$], rs4322490 [$P = 0.032$], and rs1133104 [$P = 0.045$]) and 1 was located in the intergenic region between *DCIR* and *FLJ165353* (rs7307734 [$P = 0.041$]). However, results of permutation testing suggested that this could be a false-positive result (empiric $P > 0.05$).

Following stratification for anti-CCP status, no association with anti-CCP–positive RA was detected. In contrast, analysis of 8 SNPs yielded a nominal allelic association with anti-CCP–negative RA, of which 1 was located in *DECTIN2* and 5 in *DCIR*, despite loss of power in the analysis (for *DCIR* marker rs1133104, OR 1.27, 95% CI 1.06–1.52, $P = 0.0073$) (Table 1 and Figure 5). The *DCIR* markers showing nominal associations were located in 3 different recombination blocks, as determined by LD mapping (Figure 5). None of the associations were significant by permutation testing (for *DCIR* marker rs1133104, empiric $P = 0.16$).

Construction of a human LD map over *APLEC*, and analysis of haplotype associations with RA. Human *DCIR* lies ~394 kb centromeric to *DLEC* and ~340 kb telomeric to *DECTIN2*, *MCL*, and *MINCLE*. Analysis of LD between the 35 genotyped markers revealed low LD between these 3 genetic loci (Figure 5), and therefore they were subjected to haplotype tests independently for determining any associations with RA. No haplotype within the recombination blocks was associated with RA, nor was there any association of a haplotype with RA stratified by anti-CCP status. However, one haplotype defined by 5 SNPs, which were located in 2 recombination blocks covering *DCIR* and a flanking region, did show an association with susceptibility to anti-CCP–negative RA (for SNPs 6, 12–14, and 20 in Figure 5, OR 1.37, 95% CI 1.12–1.67, $P = 0.0019$), which was supported by the findings of permutation testing (empiric $P = 0.045$). The frequency of the haplotype CAAAA was 0.20 in healthy control subjects and 0.25 in patients with anti-CCP–negative RA. With regard to the haplotype CGATC, although the results of permutation testing did not support our findings ($P = 0.086$), this haplotype showed a tendency to appear protective, with a frequency of 0.17 in control subjects and 0.13 in patients (OR 0.71, 95% CI 0.56–0.90, $P = 0.0037$).

DISCUSSION

Many QTLs, in addition to the MHC, have been identified in polygenic rodent RA models, but the majority have not been reproduced in congenic strains. Only 1 QTL has previously been position mapped to a few genes, with some evidence for a single causal genetic variation in *Ncf1* but without excluding the influence of other types of genes (29). No reports exist on the potential association of *NCF1* with RA.

Herein we provide the first example of position mapping of a single type of gene underlying polygenic experimental arthritis, with the identification of *APLEC* as a novel genetic factor that regulates arthritis susceptibility in the rat and with confirmation of this finding in a congenic strain. This sets the stage for further research that may provide clues to the pathogenesis of arthritis and result in strategies for disease prevention and treatment. Theoretically, human disease pathways involving *APLEC*-encoded receptors could be important, although genetic variations in human *APLEC* are not associated with disease. However, our present results do indicate that *APLEC* and the human *APLEC* gene *DCIR* are genetically associated with anti-CCP–negative RA. In addition to encouraging positional identification of

other rodent genes that could confer susceptibility to polygenic arthritis, as well as prompting further evaluations in human RA, our results provide a strong incentive to investigate the role of *APLEC* in other rheumatic diseases.

The *APLEC*-encoded receptors are type II transmembrane proteins that are preferentially expressed on neutrophils and APCs, i.e., DCs, macrophages, and B cells. Their extracellular part contains a lectin-like domain with preserved amino acid residues involved in calcium-dependent carbohydrate binding, leading to their classification as group II CLECSF members. This receptor type has been implicated in cellular adhesion and migration, microbial pattern recognition, antigen uptake, T cell costimulation, and signal transduction (30,31). Structural features as well as functional studies indicate, however, that the presently mapped group II CLECSF receptors transduce activating or inhibiting signals. However, the present uncertainty as to receptor function precludes direct interpretation and extrapolation of our genetic results to disease pathways. Further study on the biologic role of *APLEC*-encoded receptors in both health and disease is therefore warranted.

In the rat, all of the Clecsf receptors are encoded from genes located in tandem within the 535-kb *APLEC* that is positioned ~6.0 Mb proximal to the natural killer cell complex (*NKC*) (7). Similar to the *NKC*-encoded killer cell lectin-like receptors (32,33), the *APLEC*-encoded receptors have structural features that are indicative of a possible regulatory role in leukocyte reactivity by opposing signaling functions. Thus, rat Dcir1 and Dcir2 carry immunoreceptor tyrosine–based inhibitory motifs (ITIMs) in their cytoplasmic domains, conveying an inhibitory function through recruitment of protein tyrosine phosphatases to phosphorylated ITIMs following receptor ligation. Functional studies of mouse Dcir1 have indeed demonstrated ITIM-dependent inhibition of B cell receptor–mediated Ca^{++} mobilization and protein tyrosine phosphorylation (34).

Conversely, Mincle has a positively charged amino acid in the transmembrane domain, suggesting that it has an activating function through associations with adapter molecules carrying immunoreceptor tyrosine–based activating motifs (ITAMs). Some of the receptors carry neither ITIMs nor charged transmembrane residues, suggesting that they may play a role other than signaling. However, human DLEC and mouse Dcar2 have been shown to activate calcium mobilization and protein tyrosine phosphorylation (35,36), although they lack a positively charged amino acid in the transmembrane domain. This indicates that there are as-yet-unidentified signaling motifs, or that signaling occurs through heterodimer formation.

Taken together, the evidence for inhibitory and activating signaling functions indicates that the receptors function by fine-tuning leukocyte responses. On this background, each and all of the *APLEC*-encoded receptors may potentially influence arthritis, either singly or in concert. Consequently, it may be difficult to assign phenotypes to single genes, especially in the rat, a genetic environment in which the genes are tightly linked. Since multiple differences in cDNA sequence and mRNA expression were detected in our experimental system, we cannot exclude any of the genes, or indeed any *APLEC* sequence, from having a potential impact on arthritis.

Nevertheless, *Dcar1* stands out as a strong candidate, with a predicted early nonsense mutation rendering it nonfunctional in the DA strain, which would then be a natural knockout for *Dcar1*. How *Dcar1* or other genes could influence arthritis development remains an enigma, largely because the ligands remain undetermined. The experimental arthritis studied herein is elicited by a single intradermal injection of Freund's incomplete adjuvant oil at the base of the tail, leading to T cell–dependent inflammation in the fore and hind paws 2 weeks later. With this temporal and spatial separation of the 2 events, the stage and site of action of dysregulated leukocytes is purely hypothetical, but it appears likely that *APLEC* influences the largely undetermined interplay between APCs and T cells in arthritis development.

Following the positional identification of *APLEC* as an arthritis determinant in the rat, we targeted the 5 homologous human *APLEC* genes *DECTIN2, MCL, MINCLE, DCIR*, and *DLEC*, with a focus on *DCIR*, since initial analysis showed an association of this gene with RA. It can be suspected that the non-MHC genes will not generate high OR values. Our present results indicate an association of *DCIR* with anti-CCP–negative RA, with the SNP rs1133104 yielding an OR of 1.27 (95% CI 1.06–1.52) and the *DCIR* haplotype yielding an OR of 1.37 (95% CI 1.12–1.67). The association between anti-CCP–negative RA and the haplotype, which combines SNPs in different recombination blocks, remained significant after permutation testing, whereas none of the associations with single SNPs remained significant.

In sum, a conservative interpretation would be that our results indicate an association, but replication is warranted. Unfortunately, we have been unable to identify a similar-sized case–control study sample. Furthermore, the nature of the variation that potentially under-

lies susceptibility to anti-CCP–negative RA remains to be determined. In addition to a polymorphism in a gene, this finding may be attributed to a variation in a regulatory region affecting transcription, splicing, or mRNA stability. Moreover, we cannot completely exclude the possibility that our results reflect an association with a variation inside *APLEC*, but in a gene or genes unrelated to the C-type lectin–like receptors. For future studies, we aim to extend our analyses to greater numbers of analyzed genetic variations and greater numbers of analyzed phenotypes.

Based on our present results we can conclude that genetic variations in the *APLEC* gene cluster are associated with arthritis susceptibility in the rat, and possibly in humans. It could be expected that *APLEC* may also be associated with other rheumatic diseases. In mice, intraperitoneal injection of the presently used arthritogenic oil can induce nuclear autoantibodies associated with systemic lupus erythematosus (SLE) (37). A link between the action of the adjuvant oil and *APLEC* is a possible mechanism of action, since production of interferon-α/β by plasmacytoid DCs is considered to be a major pathophysiologic factor in human SLE, with DLEC, also known as BDCA2, being a specific marker for this cell type. In fact, it has been shown that ligation of DLEC suppresses the induction of interferon-α/β production (35). In the context of oil-induced phenotypes, it is interesting that a Swedish case–control study on environmental factors demonstrated an association between occupational exposure to certain mineral oils and increased risk of RA (38). Apparently, oil-induced arthritides in the rat represent "searchlight" models for the study of inflammatory responses, in which similarities to RA can be found with regard to clinical manifestations (13,23,39), disease-inducing agents (38,40), and disease-regulating genetic factors as demonstrated previously for the *MHC* and herein with indications for *APLEC*.

However, the importance of *APLEC* most likely goes beyond influencing oil-induced phenomena and rheumatic diseases, as deduced from several lines of evidence. First, polyarthritis can be induced in rats by many different types of immunostimulants other than oils, including many agents of microbial origin (41). Second, the presently used immunostimulating oil is used routinely together with autoantigens to disrupt immune system tolerance and induce experimental correlates of various human autoimmune diseases. Consequently, the genes that influence oil-induced responses also represent natural candidates for association with infectious diseases and with autoimmune diseases (5). Therefore, it is interesting that QTLs for arthritis and multiple other immunopathologic conditions do cluster at the *Oia2/APLEC* locus in the rat, mouse, and humans (5,6,42–47). The clustering of QTLs could therefore reflect the shared presence of genes with a general impact on the immune system and its manifestations.

With their expression on leukocytes, and as likely regulators of cellular activity, the identified *APLEC*-encoded receptors represent interesting candidate risk gene products. Efforts to delineate how individual and collective *APLEC* polymorphisms translate to different immunopathologic conditions in different species have therefore been initiated. Our preliminary results regarding the influence of *APLEC* suggest an influence on a wide range of phenotypes that are related to immunity and inflammation, and investigations are currently under way to explore these issues.

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AUTHOR CONTRIBUTIONS

Dr. Lorentzen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Lorentzen, Flornes, Eklöw, Bäckdahl, Guo, Dissen, Alfredsson, Klareskog, Padyukov, Fossum.

Acquisition of data. Lorentzen, Flornes, Eklöw, Bäckdahl, Ribbhammar, Guo, Smolnikova, Seddighzadeh, Brookes, Alfredsson, Klareskog.

Analysis and interpretation of data. Lorentzen, Flornes, Eklöw, Bäckdahl, Ribbhammar, Guo, Dissen, Padyukov, Fossum.

Manuscript preparation. Lorentzen, Flornes, Eklöw, Bäckdahl, Ribbhammar, Guo, Dissen, Brookes, Alfredsson, Klareskog, Padyukov, Fossum.

Statistical analysis. Lorentzen, Eklöw, Bäckdahl, Guo, Padyukov.

ROLE OF THE STUDY SPONSORS

The study sponsors, including Pfizer Pharmaceutical, were not involved in the study set-up, data collection, or analysis and interpretation of the data, and had no influence on the publishing of the data.

REFERENCES

1. Silman AJ, MacGregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, et al. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. Br J Rheumatol 1993;32:903–7.
2. Deighton CM, Walker DJ, Griffiths ID, Roberts DF. The contribution of HLA to rheumatoid arthritis. Clin Genet 1989;36: 178–82.
3. Gregersen PK, Silver J, Winchester RJ. The shared epitope

hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987;30: 1205–13.
4. Jansson AM, Jacobsson L, Luthman H, Lorentzen JC. Susceptibility to oil-induced arthritis is linked to Oia2 on chromosome 4 in a DA(DA × PVG.1AV1) backcross. Transplant Proc 1999;31: 1597–9.
5. Lorentzen JC, Glaser A, Jacobsson L, Galli J, Fakhrai-rad H, Klareskog L, et al. Identification of rat susceptibility loci for adjuvant-oil-induced arthritis. Proc Natl Acad Sci U S A 1998;95: 6383–7.
6. Ribbhammar U, Flornes L, Backdahl L, Luthman H, Fossum S, Lorentzen JC. High resolution mapping of an arthritis susceptibility locus on rat chromosome 4, and characterization of regulated phenotypes. Hum Mol Genet 2003;12:2087–96.
7. Flornes LM, Bryceson YT, Spurkland A, Lorentzen JC, Dissen E, Fossum S. Identification of lectin-like receptors expressed by antigen presenting cells and neutrophils and their mapping to a novel gene complex. Immunogenetics 2004;56:506–17.
8. Van Gaalen FA, van Aken J, Huizinga TW, Schreuder GM, Breedveld FC, Zanelli E, et al. Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCPs) influences the severity of rheumatoid arthritis. Arthritis Rheum 2004;50:2113–21.
9. Klareskog L, Stolt P, Lundberg K, Kallberg H, Bengtsson C, Grunewald J, et al, and the Epidemiological Investigation of Rheumatoid Arthritis Study Group. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA–DR (shared epitope)–restricted immune reactions to autoantigens modified by citrullination. Arthritis Rheum 2006;54:38–46.
10. Woon PY, Osoegawa K, Kaisaki PJ, Zhao B, Catanese JJ, Gauguier D, et al. Construction and characterization of a 10-fold genome equivalent rat P1-derived artificial chromosome library. Genomics 1998;50:306–16.
11. Dissen E, Ryan JC, Seaman WE, Fossum S. An autosomal dominant locus, Nka, mapping to the Ly-49 region of a rat natural killer (NK) gene complex, controls NK cell lysis of allogeneic lymphocytes. J Exp Med 1996;183:2197–207.
12. Berg SF, Dissen E, Westgaard IH, Fossum S. Molecular characterization of rat NKR-P2, a lectin-like receptor expressed by NK cells and resting T cells. Int Immunol 1998;10:379–85.
13. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
14. Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, et al. Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. Ann Rheum Dis 2003;62: 835–41.
15. Jobs M, Howell WM, Stromqvist L, Mayr T, Brookes AJ. DASH-2: flexible, low-cost, and high-throughput SNP genotyping by dynamic allele-specific hybridization on membrane arrays. Genome Res 2003;13:916–24.
16. Howell WM, Jobs M, Gyllensten U, Brookes AJ. Dynamic allele-specific hybridization: a new method for scoring single nucleotide polymorphisms. Nat Biotechnol 1999;17:87–8.
17. Prince JA, Feuk L, Howell WM, Jobs M, Emahazion T, Blennow K, et al. Robust and accurate single nucleotide polymorphism genotyping by dynamic allele-specific hybridization (DASH): design criteria and assay validation. Genome Res 2001;11:152–62.
18. Fredman D, Jobs M, Stromqvist L, Brookes AJ. DFold: PCR design that minimizes secondary structure and optimizes downstream genotyping applications. Hum Mutat 2004;24:1–8.
19. Jobs M, Howell WM, Brookes AJ. Creating arrays by centrifugation. Biotechniques 2002;32:1322–4, 1326, 1329.
20. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21: 263–5.
21. Holm BC, Lorentzen JC, Bucht A. Adjuvant oil induces waves of arthritogenic lymph node cells prior to arthritis onset. Clin Exp Immunol 2004;137:59–64.
22. Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet 1999;8:1893–900.
23. Carlson BC, Jansson AM, Larsson A, Bucht A, Lorentzen JC. The endogenous adjuvant squalene can induce a chronic T-cell-mediated arthritis in rats. Am J Pathol 2000;156:2057–65.
24. Bates EE, Fournier N, Garcia E, Valladeau J, Durand I, Pin JJ, et al. APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif. J Immunol 1999;163:1973–83.
25. Arce I, Martinez-Munoz L, Roda-Navarro P, Fernandez-Ruiz E. The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor. Eur J Immunol 2004;34:210–20.
26. Matsumoto M, Tanaka T, Kaisho T, Sanjo H, Copeland NG, Gilbert DJ, et al. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. J Immunol 1999;163:5039–48.
27. Arce I, Roda-Navarro P, Montoya MC, Hernanz-Falcon P, Puig-Kroger A, Fernandez-Ruiz E. Molecular and genomic characterization of human DLEC, a novel member of the C-type lectin receptor gene family preferentially expressed on monocyte-derived dendritic cells. Eur J Immunol 2001;31:2733–40.
28. Kanazawa N, Tashiro K, Miyachi Y. Signaling and immune regulatory role of the dendritic cell immunoreceptor (DCIR) family lectins: DCIR, DCAR, dectin-2 and BDCA-2. Immunobiology 2004;209:179–90.
29. Olofsson P, Holmberg J, Tordsson J, Lu S, Akerstrom B, Holmdahl R. Positional identification of Ncf1 as a gene that regulates arthritis severity in rats. Nat Genet 2003;33:25–32.
30. Cambi A, Figdor CG. Dual function of C-type lectin-like receptors in the immune system. Curr Opin Cell Biol 2003;15:539–46.
31. Geijtenbeek TB, van Vliet SJ, Engering A, BA 't Hart, van Kooyk Y. Self- and nonself-recognition by C-type lectins on dendritic cells. Annu Rev Immunol 2004;22:33–54.
32. Weis WI, Taylor ME, Drickamer K. The C-type lectin superfamily in the immune system [review]. Immunol Rev 1998;163:19–34.
33. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex [review]. Nat Rev Immunol 2003;3: 304–16.
34. Kanazawa N, Okazaki T, Nishimura H, Tashiro K, Inaba K, Miyachi Y. DCIR acts as an inhibitory receptor depending on its immunoreceptor tyrosine-based inhibitory motif. J Invest Dermatol 2002;118:261–6.
35. Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon α/β induction. J Exp Med 2001;194:1823–34.
36. Kanazawa N, Tashiro K, Inaba K, Miyachi Y. Dendritic cell immunoactivating receptor, a novel C-type lectin immunoreceptor, acts as an activating receptor through association with Fc receptor γ chain. J Biol Chem 2003;278:32645–52.
37. Satoh M, Kuroda Y, Yoshida H, Behney KM, Mizutani A, Akaogi J, et al. Induction of lupus autoantibodies by adjuvants. J Autoimmun 2003;21:1–9.
38. Sverdrup B, Kallberg H, Bengtsson C, Lundberg I, Padyukov L, Alfredsson L, et al. Association between occupational exposure to mineral oil and rheumatoid arthritis: results from the Swedish EIRA case-control study. Arthritis Res Ther 2005;7:R1296–303.
39. Holmdahl R, Lorentzen JC, Lu S, Olofsson P, Wester L, Holmberg J, et al. Arthritis induced in rats with nonimmunogenic adjuvants as models for rheumatoid arthritis [review]. Immunol Rev 2001;184:184–202.
40. Kleinau S, Erlandsson H, Holmdahl R, Klareskog L. Adjuvant oils

induce arthritis in the DA rat. I. Characterization of the disease and evidence for an immunological involvement. J Autoimmun 1991;4:871–80.
41. Lorentzen JC. Identification of arthritogenic adjuvants of self and foreign origin. Scand J Immunol 1999;49:45–50.
42. Dahlman I, Lorentzen JC, de Graaf KL, Stefferl A, Linington C, Luthman H, et al. Quantitative trait loci disposing for both experimental arthritis and encephalomyelitis in the DA rat; impact on severity of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis and antibody isotype pattern. Eur J Immunol 1998;28:2188–96.
43. Becker KG, Simon RM, Bailey-Wilson JE, Freidlin B, Biddison WE, McFarland HF, et al. Clustering of non-major histocompatibility complex susceptibility candidate loci in human autoimmune diseases. Proc Natl Acad Sci U S A 1998;95:9979–84.
44. Cornelis F, Faure S, Martinez M, Prud'homme JF, Fritz P, Dib C, et al. New susceptibility locus for rheumatoid arthritis suggested by a genome-wide linkage study. Proc Natl Acad Sci U S A 1998;95:10746–50.
45. Jawaheer D, Seldin MF, Amos CI, Chen WV, Shigeta R, Monteiro J, et al. A genomewide screen in multiplex rheumatoid arthritis families suggests genetic overlap with other autoimmune diseases. Am J Hum Genet 2001;68:927–36.
46. Osorio y Fortea J, Bukulmez H, Petit-Teixeira E, Michou L, Pierlot C, Cailleau-Moindrault S, et al, for the European Consortium on Rheumatoid Arthritis Families. Dense genome-wide linkage analysis of rheumatoid arthritis, including covariates. Arthritis Rheum 2004;50:2757–65.
47. Fisher SA, Lanchbury JS, Lewis CM. Meta-analysis of four rheumatoid arthritis genome-wide linkage studies: confirmation of a susceptibility locus on chromosome 16. Arthritis Rheum 2003; 48:1200–6.